Poly(β-phosphorylated nitrones): preparation and characterisation of a new class of spin trap

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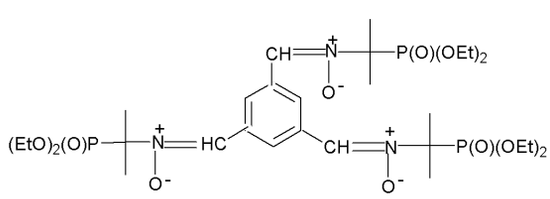
Four new poly(β-phosphorylated nitrones), the 1,3,5-tris[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (TN) **1**, the 1,3-bis[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (MDN) **2**, the 1,4-bis[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (PDN) **3**, and the 1,2-bis[*N*-(1-diethylphosphono)-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (ODN) **4**, derived from the mono-nitrone *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) **5**, were synthesised. The capacity of **1**–**4** to act as spin-trapping agents was investigated in phosphate buffers at pH 5.8 and 7.2. Complex EPR spectra were obtained for the spin adducts with *ortho*-dinitrone **4**. The three other compounds efficiently trapped superoxide and several carbon-centred radicals, giving mono-spin adducts, although only weak signals were obtained with the hydroxyl radical. When the spin trap concentration was kept below 1 mmol dm^{-3}, the formation of di-radicals also occurred. The half-lives of the superoxide spin adducts of **1**–**3** were in the range of 5–12 min and did not change significantly between pH 5.8 and 7.2. A competitive kinetic study showed that the trinitrone **1** trapped the methyl radical 1.9 times more rapidly than either α-(1-oxidopyridin-1-ium-4–yl)-*N*-*tert*-butylnitrone (POBN) or 5-diethoxyphosphoryl-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DEPMPO) at pH 7.2.

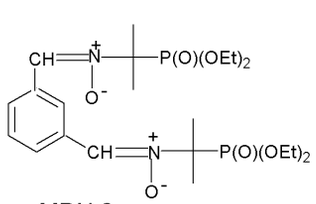
Introduction

Oxygen-centred radicals can be produced by many biological mechanisms and could be involved in many human pathologies such as cancer, atherosclerosis or heart attack.[1] The technique of spin trapping of short-lived radical intermediates by nitrones has become a valuable tool in the study of radical processes occurring in chemical or biochemical environments.[2] However, many *in vivo* applications of spin traps could be restricted either by the bio-distribution of these compounds or by their toxicity, which often prevents the use of nitrone concentrations that are sufficiently high to permit detection of low levels of free radicals. To overcome this problem, much effort has been devoted to the elaboration of new nitrones that could give more persistent spin adducts. In this field, the presence of an electron-withdrawing group, such as a phosphorylated group[3,4] or a carboxy ester group,[5] in the β-position to the nitrone functionality has been shown to significantly increase the lifetime of the superoxide adduct. Another way to obtain more efficacious nitrones for the detection of free radicals is to increase their spin-trapping rate. In this perspective, we have synthesised four poly(β-phosphorylated) nitrones: the 1,3,5-tris[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (TN) **1**, the 1,3-bis[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (MDN) **2**, the 1,4-bis[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (PDN) **3**, and the 1,2-bis[*N*-(1-diethylphosphono)-1-methylethyl)-*N*-oxidoiminiomethyl]benzene (ODN) **4**. The corresponding mono-nitrone, the *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) **5**, has been extensively studied in recent years and possesses interesting spin-trapping properties.[4c,6]

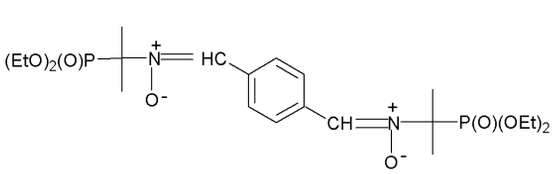
Our aim was to obtain a new class of spin trap with better trapping effectiveness as a result of the presence of two or three nitrone functional groups in these molecules. The lipophilicity of these new compounds has been determined by measuring



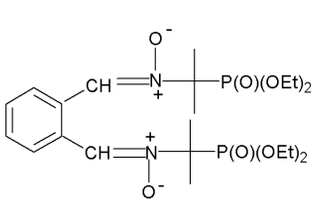
TN **1**



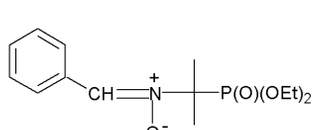
MDN **2**



PDN **3**



ODN **4**



PPN **5**

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Table 1 Partition coefficients of compounds **1**–**4** in *n*-octanol–phosphate buffer (0.1 mol dm^{-3})

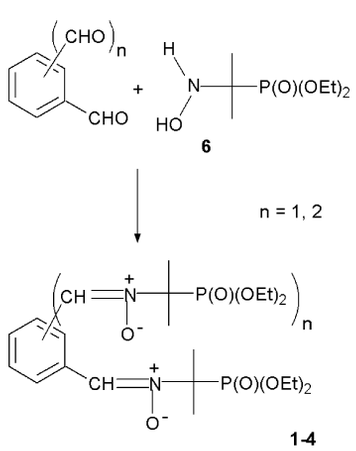
Spin trap	K_p
TN **1**	>300
MDN **2**	20
PDN **3**	65
ODN **4**	11.5

their octanol–phosphate buffer partition coefficient. Their spin-trapping capacity has been surveyed by using them to trap oxygen and carbon-centred radicals in aqueous media. The decay kinetics of the superoxide spin adducts and the trapping rate of the methyl radical have been studied in particular. The first results of this work are presented in this paper.

Results and discussion

Synthesis

Diethyl [1-(hydroxyamino)-1-methylethyl]phosphonate **6** was first synthesised following the method of Petrov *et al.*[7] and purified as previously described.[6c] Then, the poly-nitrones **1**–**4** were prepared in a one-step reaction by condensing **6** with the corresponding poly-aldehyde, as shown in Scheme 1. The



Scheme 1 Synthesis of compounds **1**–**4**.

requisite tri-aldehyde for the synthesis of poly-nitrone **1**, *i.e.* 1,3,5-triformylbenzene, was prepared beforehand by following a two-step synthesis that has been described previously.[8] Nitrones **1**, **3** and **4** were purified by recrystallisation, while **2** was purified by chromatography, as described in the Experimental section. Following this procedure, compounds **1**–**4** were obtained in high purity, and in reasonable yields.

Lipophilicity

Of the methods available to evaluate the lipophilicity of a spin trap, one of the most often used is the determination of its *n*-octanol–water or *n*-octanol–phosphate buffer partition coefficient, *i.e.* K_p, by UV spectroscopy.[9] However, we found that this method could be inaccurate in some cases and we have previously described another technique in which HPLC was used to determine the spin-trap concentration in both the octanolic and aqueous phases.[6c,10] Following this technique, partition coefficients (K_p) in *n*-octanol–phosphate buffer (0.1 mol dm^{-3}, pH 7) were evaluated for compounds **1**–**4**. As can be seen from Table 1, **1**–**4** were found to be more lipophilic than 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO, K_p = 0.1),[9d] α-phenyl-*N*-*tert*-butylnitrone (PBN, K_p = 15)[9d] and PPN **5** (K_p = 10.2).[6c] Despite its high lipophilicity, **1** showed rather high solubility in aqueous media. Compound **3** was less easily dissolved in 0.1 mol dm^{-3} phosphate buffer, but solutions containing 15 mmol dm^{-3} PDN could be prepared without major difficulties.

Spin trapping

In order to appreciate the potential of our new compounds in the detection of short-lived radicals, a series of free radicals was trapped in buffered solutions by each one of the poly-nitrones. In order to simplify the notation, the aminoxyl obtained by trapping the radical $R^{\bullet}$ by a spin trap N will be noted N–R.

Regardless of the radical trapped, we always observed complex EPR spectra with ODN **4**. For example, the signal obtained by carrying out a Fenton reaction in the presence of methanol and ODN is represented in Fig. 1a. Its simulation (Fig. 1b) was achieved by considering the presence of four

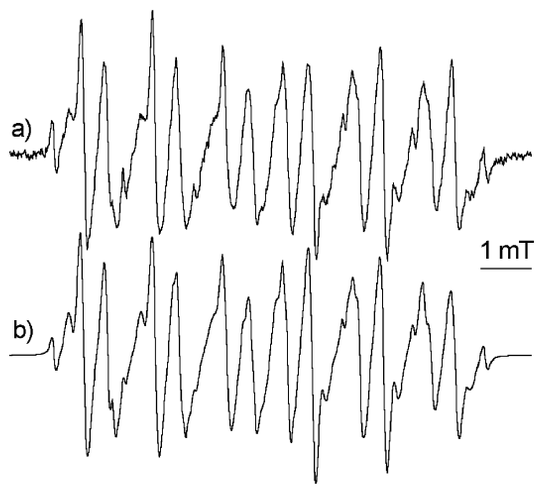
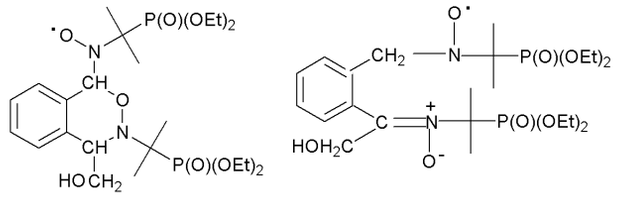


Fig. 1 a) EPR signal obtained by carrying out a Fenton reaction in the presence of methanol (15%) and ODN **4** (5 mmol dm^{-3}) at pH 7.2. The following recording conditions were used: non-saturating microwave power, 10 mW; modulation amplitude, 10^{-2} mT; receiver gain, 5×10^3; scan time, 335.5 s; time constant, 327.68 ms. b) Simulation of signal a) obtained by considering the presence of four aminoxyl radicals, with the following parameters: for the first (64.1%), a_H = 0.49, a_P = 4.12 and a_N = 1.45 mT; for the second (13.1%), a_H = 0.90, a_P = 4.16 and a_N = 1.45 mT; for the third (8.7%), a_H = 1.20 (2H), a_P = 3.42 and a_N = 1.42 mT; for the fourth (14.1%), a_H = 0.20, a_P = 4.31 and a_N = 1.43 mT.

aminoxyl radicals. Although one could hardly claim to identify these species solely on the basis of their hyperfine coupling constants (hfcc's), a hypothesis can be proposed for their assignments. The a_H values determined for the first (64.1%, a_H = 0.49, a_P = 4.12 and a_N = 1.45 mT) and more particularly for the second species (13.1%, a_H = 0.90, a_P = 4.16 and a_N = 1.45 mT) were found to be significantly higher than those usually observed for free radical adducts of linear nitrones, such as PBN or PPN. This could suggest that these two aminoxyl radicals are cyclic. We thus made the assumption that they might correspond to the two diastereoisomers of **7**, formed from the spin adduct ODN–CH_2OH by an intramolecular spin-trapping reaction. The third species (8.7%, a_H = 1.20 (2H), a_P = 3.42 and a_N = 1.42 mT) exhibited a coupling of the unpaired electron with two equivalent hydrogen nuclei. In addition, a_H was found to be rather high, as previously observed in the case of hydrogen radical adducts of PPN-type nitrones.[6c] On the basis of these observations, we thought that this aminoxyl radical could correspond to **8**, which may be obtained from ODN–CH_2OH by intramolecular hydrogen transfer. Careful analysis of the EPR parameters of the fourth species (14.1%, a_H = 0.20, a_P = 4.31 and a_N = 1.43 mT) did not provide enough information about the nature of this radical. Note also that the structures proposed for the three other ODN-derived radicals remain hypothetical. Much more evidence would be needed to assign these species unambiguously and to determine



7 8

the mechanisms responsible for their formation. However, similar results were obtained with ODN whatever the nature of the radical generated. The resulting EPR spectra always showed the simultaneous presence of at least three different paramagnetic species. This could indicate that the proximity of the two nitrone functions on the aromatic ring would favour intramolecular reactions, thereby generating new aminoxyl radicals. Though the species thus formed were not clearly identified, these results definitely showed that ODN **4** did not possess any interesting spin-trapping properties, and we decided to remove it from the rest of our study.

In the presence of compounds **1**–**3** (5 mmol dm^{-3}), and whatever the identity of the radical trapped, we always observed mono-spin adducts that gave simple EPR signals with twelve lines, due to hyperfine couplings of the unpaired electron with the nitrogen, the phosphorus and the β-hydrogen nuclei. Note, however, that the formation of poly-spin adducts might occur when very low spin-trap concentrations are used (below 1 mmol dm^{-3}), as discussed later.

The carbon-centred radicals $^{\bullet}CH_3$, $^{\bullet}CH_2OH$ and $^{\bullet}COO^-$ were generated in aqueous media using a standard Fenton system in the presence of **1**–**3** and of DMSO, methanol or sodium formate, respectively, as described in the Experimental section. Poly-nitrones **1**–**3** were found to trap efficiently every kind of carbon-centred radical, yielding very persistent spin adducts, which lasted for several hours (see Fig. 2a).

When $^{\bullet}OH$ was produced in the presence of **1**–**3**, only weak spectra of spin adducts were recorded (see Fig. 2b). They were completely inhibited in the presence of catalase (400 unit cm^{-3}), and were attributed to the hydroxyl spin adducts. The same weak signals have also been obtained by nucleophilic addition of water to **1**–**3** in the presence of ferric ions, followed by autoxidation. As mentioned earlier in the case of various linear PBN-type traps,[6d,11] the hydroxyl radical adducts of **1**–**3** seem to decompose rapidly in aqueous media, and these nitrones cannot be considered as efficient spin traps for $^{\bullet}OH$ radicals.

More interesting results were obtained when superoxide was produced by either a xanthine–xanthine oxidase (X–XO) system or a light–riboflavin–electron donor (LRED) system in the presence of **1**–**3**. Regardless of the superoxide generator, rather intense EPR spectra were recorded at both pH 5.8 and 7.2. To illustrate this, the EPR signal obtained at pH 7.2 by generating superoxide with the X–XO system in the presence of MDN (5 mmol dm^{-3}) is shown in Fig. 2c. We verified that the formation of these superoxide adducts was completely inhibited by superoxide dismutase (600–1200 unit cm^{-3}) and was unaffected by catalase (600 unit cm^{-3}). When using the LRED system, a small amount of an unidentified carbon-centred radical adduct was also observed. The formation of this radical (denoted $^{\bullet}C$) is inherent in this superoxide generator and has been reported for other nitrones, such as 5-diethoxyphosphoryl-5-methyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DEPMPO), DMPO, or PPN **5**.[4b,4c,12]

The hfcc's a_N, a_P and a_H of all the various spin adducts of compounds **1**–**3**, obtained by simulating experimental spectra, are listed in Table 2. Because of the presence of a large phosphorus coupling, all of these spectra are very characteristic of the species trapped. This spectral uniqueness should be regarded as a major advantage of ^{31}P-labelled spin traps, notably in the linear nitrone series. For example, the difference in

Table 2 EPR hyperfine coupling constants for various spin adducts of TN **1**, MDN **2** and PDN **3** in 0.1 mol dm^{-3} phosphate buffer, pH 7.2

Spin trap	Radical trapped	a_N/mT	a_H/mT	a_P/mT
TN **1**	$^{\bullet}CH_3$	1.46	0.29	4.62
	$^{\bullet}CH_2OH$	1.44	0.27	4.29
	$^{\bullet}CO_2^-$	1.43	0.30	4.91
	$^{\bullet}OH$	1.47	0.18	4.41
	$^{\bullet}O_2^-$	1.34	0.16	4.24
	$^{\bullet}C$[a]	1.50	0.23	4.50
MDN **2**	$^{\bullet}CH_3$	1.49	0.32	4.65
	$^{\bullet}CH_2OH$	1.45	0.30	4.29
	$^{\bullet}CO_2^-$	1.45	0.36	4.93
	$^{\bullet}OH$	1.44	0.29	4.32
	$^{\bullet}O_2^-$	1.35	0.18	4.21
	$^{\bullet}C$[a]	1.50	0.28	4.40
PDN **3**	$^{\bullet}CH_3$	1.49	0.32	4.66
	$^{\bullet}CH_2OH$	1.46	0.30	4.30
	$^{\bullet}CO_2^-$	1.45	0.37	4.92
	$^{\bullet}OH$	1.44	0.26	4.43
	$^{\bullet}O_2^-$	1.34	0.18	4.23
	$^{\bullet}C$[a]	1.47	0.35	4.40

[a] $^{\bullet}C$ corresponds to an unidentified carbon-centred radical produced by the LRED superoxide generator.

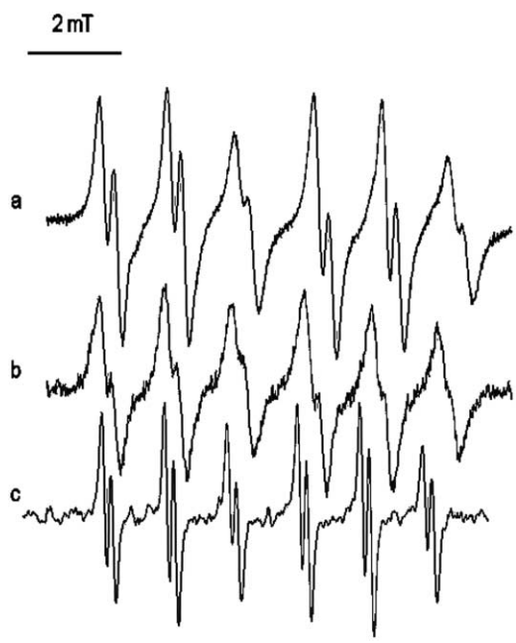


Fig. 2 a) EPR spectrum of TN–CH_3 obtained by carrying out a Fenton reaction in the presence of dimethyl sulfoxide (15%) and **1** (5 mmol dm^{-3}) at pH 7.2. b) EPR spectrum of PDN–OH, obtained by using a Fenton system to produce $^{\bullet}OH$ radicals in the presence of **3** (5 mmol dm^{-3}) at pH 5.8. c) EPR spectrum of MDN–O_2H obtained in a pH 7.2 buffer by generating superoxide with a xanthine–xanthine oxidase system in the presence of **2** (5 mmol dm^{-3}). The following recording conditions were used: non-saturating microwave power, 10 mW; modulation amplitude, a) 0.2 mT, b) 0.15 mT, c) 0.125 mT; scan time, a) and b) 168 s, c) 180 s; time constant, a) 41 ms, b) 164 ms, c) 250 ms; receiver gain, a) 5×10^5, b) 2.5×10^6, c) 8×10^4.

the total width of the EPR spectrum of the superoxide and hydroxyl adducts was always found to be higher than 0.4 mT with PPN or with **1**–**3**, and lower than 0.1 mT with PBN. Thus, the use of phosphorylated nitrones could avoid mistakes in spin-adduct assignments. Note that the hfcc with the nitrogen, a_N, is also very characteristic in the superoxide adducts of **1**–**3**: its value is 1.34–1.35 mT, whereas this coupling is significantly higher in the other cases.

As mentioned above, double-trapping might have occurred when low poly-nitrone concentrations were used. The

formation of poly-adducts, likely di-adducts, altered the spectra recorded, notably by greatly broadening the lines. To illustrate this, various spectra obtained by generating $^{\bullet}CO_2^-$ at pH 7.2 in the presence of TN **1** at 10 and 0.375 mmol dm^{-3} are presented in Fig. 3. At rather high TN concentration (10 mmol dm^{-3}), the

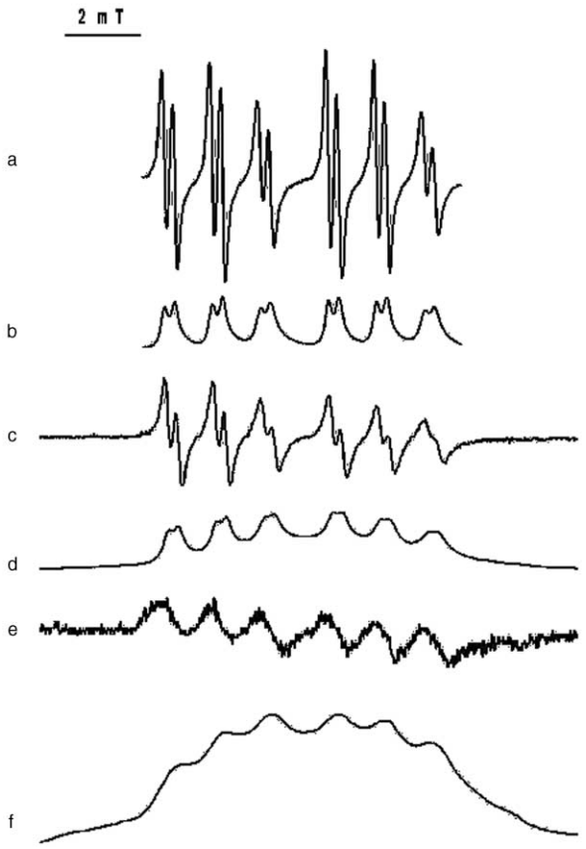


Fig. 3 a) EPR spectrum obtained by generating $^{\bullet}CO_2^-$ radicals in the presence **1** (10 mmol dm^{-3}) at pH 7.2. b) First integration of a). c) EPR spectrum obtained by generating $^{\bullet}CO_2^-$ radicals in the presence **1** (0.375 mmol dm^{-3}) at pH 7.2, and recorded 1 min after the trapping reaction had begun. d) First integration of c). e) Same as in c), but recorded 2.4 min after the beginning of the trapping reaction. f) First integration of e). The following recording conditions were used: non-saturating microwave power, 10 mW; modulation amplitude, a) 0.1 mT, c) and e) 0.15 mT; scan time, 84 s; time constant, 164 ms; receiver gain, 20000.

proportion of poly-adduct formed was much too weak to modify the spectral shape. The signal obtained and its first integration (see Fig. 3a and Fig. 3b, respectively) corresponded almost exclusively to TN–CO_2^-. When the poly-nitrone concentration was decreased to 0.375 mmol dm^{-3}, the signal represented in Fig. 3c was first recorded 1 min after the trapping reaction had begun. It shows an evolution in the line shape over the course of the recording. The first part of the spectrum is quite similar to that given in Fig. 3a, but the last lines are much broader. In the beginning of the trapping reaction, the proportion of di-adduct formed was much too weak to significantly modify the spectral shape. Then, the amount of TN–CO_2^- rapidly became sufficient to compete with TN for $^{\bullet}CO_2^-$ trapping, and the concentration of di-adduct formed increased. This was also translated into a significant modification in the base line on the first integration of the spectrum (see Fig. 3d). The spectrum presented in Fig. 3e, which exhibits much broader lines than that of the mono-adduct TN–CO_2^-, was recorded from the same sample 1.4 min later. First integration of this signal (see Fig. 3f) led to a bell-shaped curve characteristic of the presence of diaminoxyl radicals with a strong electron exchange coupling and in which the distance between the two unpaired electrons is rather short. In this case, the electron spin–electron spin dipolar interaction is often sufficiently high to greatly broaden the lines, which overlap, thereby yielding a one-line EPR signal.[13] Thus, the signal shown in Fig. 3e suggests that the medium could contain mainly the di-adduct, with maybe a small amount of the mono-adduct TN–CO_2^-. Note that it could also correspond to various conformers of the di-radicals formed from **1**–**3**, exhibiting different electron exchange couplings. Unfortunately, the literature lacks references on diaminoxyl radical EPR spectra exhibiting hyperconjugative couplings with hydrogen and/or phosphorus nuclei. Spectral analysis of the signal in Fig. 3e is obviously a complex problem that would need an advanced theoretical study, and we were not able to obtain further information on the EPR parameters of the di-radicals that could be formed from **1**–**3**. Note, however, that the presence of a significant amount of di-radical was never detected when poly-nitrone concentrations of at least 1 mmol dm^{-3} were used, regardless of the radical trapped. Therefore, the possibility of double-trapping should not be considered as a drawback of these poly-nitrones.

Superoxide adduct decay

The X–XO system was chosen to study the decay of poly-nitrone superoxide adducts at pH 5.8 and 7.2. Superoxide was first produced in the presence of **1**, **2** or **3** (5 mmol dm^{-3}), and the superoxide adduct formation was stopped by adding superoxide dismutase (SOD, 1200 unit cm^{-3}) 10 min after the reaction had begun. Then, an EPR spectrum was immediately recorded over a large scan time (335 or 671 s) in order to observe the adduct decrease in a single spectrum (see Fig. 4a).

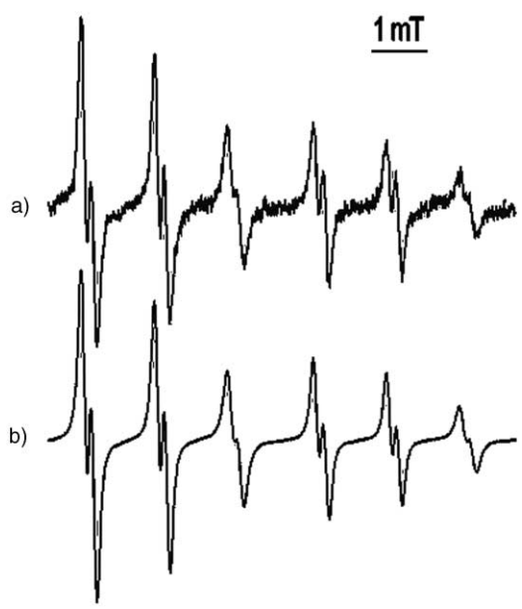


Fig. 4 a) Experimental EPR spectrum of TN–O_2H obtained by generating superoxide during 10 min with the xanthine–xanthine oxidase system in the presence of 10 mmol dm^{-3} TN at pH 5.8, and recorded after addition of SOD. The following recording conditions were used: non-saturating microwave power, 10 mW; modulation amplitude, 0.15 mT; receiver gain, 9×10^5; scan time, 671 s; time constant, 655.36 ms. b) Simulation of a) performed with the following parameters: hfcc's, $a_H = 0.16$, $a_P = 4.24$, $a_N = 1.34$ mT; first-order decay rate constant, $k_D = 2.17 \times 10^{-3}$ s^{-1}.

The spectra obtained with **1**–**3** were simulated with the computer program of Rockenbauer and Korecz,[14] which permitted us to determine the first-order rate constant k_D for the decay of superoxide adducts (see Fig 4b). The values obtained are listed in Table 3, along with the corresponding half-lives $t_{½}$. Although it is more usual to follow the decrease in one spectral line in order to study the superoxide adduct decay, the method we used is more easily implemented and avoids problems due to

Table 3 First-order rate constants (k_D) and half-life times ($t_{½}$) determined for the decay of the superoxide spin adduct for TN **1**, MDN **2** and PDN **3** in 0.1 mol dm^{-3} phosphate buffer, pH 5.8 and 7.2

	pH 5.8		pH 7.2	
Spin adduct	$k_D/10^{-3}$ s^{-1}	$t_{½}$/min	$k_D/10^{-3}$ s^{-1}	$t_{½}$/min
TN–O_2H	2.17	5.3	2.22	5.2
MDN–O_2H	1.37	8.4	1.75	6.6
PDN–O_2H	1.00	11.6	1.10	10.5

field stability or to modification in the line shape during the course of the experiment.

The half-lives of the superoxide adducts of **1**–**3** are in the range of 5–12 min. Contrary to what was observed with cyclic spin traps, DEPMPO, DMPO or 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (EMPO),[4b,5a] they did not change significantly between pH 5.8 and 7.2. Although DEPMPO–O_2H remains the most persistent superoxide adduct ($t_{½}$ = 13 min at pH 7),[4b] the superoxide adducts of the poly-nitrones studied, and notably PDN–OOH, were found to be more stable than DMPO–OOH ($t_{½}$ = 50 s at pH 7),[4b,12] EMPO–OOH ($t_{½}$ = 4.8 min at pH 7),[5a] and PPN–OOH ($t_{½}$ = 5.1 min at pH 5.6).[4c]

$^{\bullet}CH_3$ trapping kinetics

As mentioned in the Introduction, poly-nitrones **1**–**3** were expected to trap free radicals more rapidly than the most commonly used mono-nitrones. The method of kinetic competition, withthecommercialα-(1-oxidopyridin-1-ium-4–yl)-*N*-*tert*-butyl-nitrone (POBN) as competitive scavenger, was employed to examine the relative effectiveness of compounds **1**–**3** and of other nitrones. We first wanted to apply this technique to superoxide trapping. However, in order to obtain reliable results by this technique, the adduct decay must be slow enough to be neglected. For this reason, we preferred to study $^{\bullet}CH_3$ trapping, the corresponding adduct being much more stable in aqueous media.

The methyl radical was generated in a phosphate buffer of pH 7.2 in the presence of POBN and of a poly-nitrone (denoted N) as described in the Experimental section. The possibility of double-trapping by the poly-nitrone was neglected, since we never observed the formation of a significant amount of poly-adducts in these experiments. The spin-trapping rate was monitored by measuring the intensity (as the signal area) of the N–CH_3 signal. Assuming a steady-state concentration of $^{\bullet}CH_3$ at the beginning of the reaction, the kinetic model can be described by eqns. (1)–(5) (Scheme 2) in which k_N and k_{POBN} represent the trapping rate constants of $^{\bullet}CH_3$ by the poly-nitrone N under consideration and by POBN, respectively. If *r* and *R* represent the trapping rate by POBN in the absence of N

$$^{\bullet}CH_3 + N \xrightarrow{k_N} N\text{-}CH_3 \quad (1)$$

$$^{\bullet}CH_3 + POBN \xrightarrow{k_{POBN}} POBN\text{-}CH_3 \quad (2)$$

$$R = k_{POBN}\,[POBN]\,[^{\bullet}CH_3] + k_N\,[N]\,[^{\bullet}CH_3] \quad (3)$$

$$r = k_{POBN}\,[POBN]\,[^{\bullet}CH_3] \quad (4)$$

$$\frac{R}{r} = 1 + \frac{k_N}{k_{POBN}}\,\frac{[N]}{[POBN]} \quad (5)$$

Scheme 2 Kinetic model for the competition between a poly-nitrone N and POBN for the trapping of the methyl radical. k_N and k_{POBN} represent the second-order rate constants for the trapping of $^{\bullet}CH_3$ by N and by POBN, respectively; *r* and *R* are the trapping rates in the absence and in the presence of N, respectively.

Table 4 Ratio of the second-order rate constants for the methyl radical trapping by various nitrones (k_N) and by POBN (k_{POBN}) at pH 7.2

Spin trap	k_N/k_{POBN}
TN **1**	1.91
MDN **2**	1.40
PDN **3**	0.91
DEPMPO	1.01
DMPO	0.63

and by both POBN and N, respectively, then eqn. (5) is obtained by dividing eqn. (3) by eqn. (4).

By plotting the *R*/*r* ratio as a function of [N]/[POBN] (kept between 0.1 and 3.33), a straight line was obtained for each poly-nitrone **1**–**3**, according to eqn. (5) (see Fig. 5).

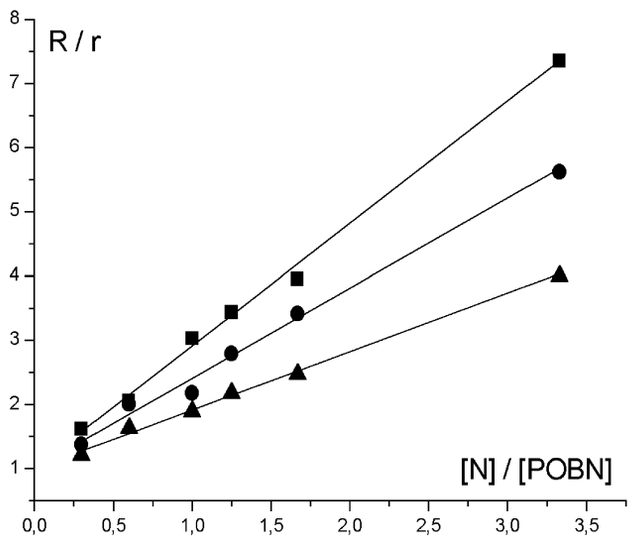


Fig. 5 Inhibition by POBN of methyl radical trapping by TN **1** (■), MDN **2** (●) and PDN **3** (▲) at pH 7.2. A Fenton system was employed in presence of methanol to produce $^{\bullet}CH_3$. The data were plotted according to eqn. (5), in which k_N and k_{POBN} represent the trapping rate constants of $^{\bullet}CH_3$ by the poly-nitrone N under consideration and by POBN, respectively, *r* and *R* being the trapping rates in the absence and in the presence of N, respectively.

The different values of the slope are equal to the ratio k_N/k_{POBN} and are listed in Table 4. These experiments were also performed with DMPO and DEPMPO, and the results obtained are given in Table 4. Unfortunately, PDN **3**, which gave the most stable superoxide adduct, was also found to be the least efficacious of the poly-nitrones with regard to the $^{\bullet}CH_3$ trapping rate. With the two other poly-nitrones, the spin-trapping rate was found to be higher. Thus, TN **1** trapped the methyl radical 1.9 times more rapidly than both POBN and DEPMPO.

Conclusion

Poly-nitrones **1**–**3** present interesting spin-trapping possibilities, and their results should be compared to those obtained with two other efficacious nitrones, DEPMPO and EMPO. The synthesis and, above all, the purification of **1**–**3** are much easier than those of DEPMPO or of EMPO. The lipophilicity of **1**–**3** is also much higher than that of both DEPMPO and EMPO. Therefore, **1**–**3** could be able to cross biomembranes and enter cells, although this hypothesis remains to be verified. The various spin adduct spectra obtained for **1**–**3** are always very simple and easily analysed, but they are characteristic of the species trapped. In contrast, the spectra obtained with either DEPMPO or EMPO are often complicated by the presence of two diastereoisomers, by conformational equilibrium, or by

long-range couplings with γ-hydrogens. With regard to hydroxyl radical trapping, cyclic nitrones are obviously much better than **1**–**3**. Note also that DEPMPO–O_2H remains the most persistent superoxide adduct, but the superoxide adducts of **1**–**3** are more stable than EMPO–O_2H. We also found that **1** and **2** trap methyl radicals 1.9 and 1.4 times more rapidly, respectively, than DEPMPO at pH 7.2. We are now trying to find a way to determine their superoxide trapping rates, and we hope that the same kind of results will be obtained with this radical.

Experimental

All chemicals and solvents were purchased from either Sigma or Aldrich companies. Enzymes were obtained from Boerhinger Mannheim Biochemica Co. The solvents were of the highest grade of purity commercially available, and used without further purification. Tri-distilled water was used to prepare aqueous media. All buffer solutions were stirred for 4 hours in the presence of a chelating acid resin (4 g dm^{-3}) to remove trace metal impurities.

Synthesis of compounds 1–4

Diethyl [1-(hydroxyamino)-1-methylethyl]phosphonate **6** was synthesised following the method of Petrov *et al.*,[7] crystallised from pentane, and recrystallised in methyl *tert*-butyl ether. Poly-nitrones **1**–**4** were prepared by heating a solution containing the corresponding poly-aldehyde (0.25 mol dm^{-3}) at 55 °C for 3 hours in the presence of the hydroxylamine **6** (0.75 and 0.5 mol dm^{-3} for the synthesis of nitrones **1** and **2**–**4**, respectively). Compounds **1**–**3** were synthesised in ethanol, while benzene was employed in the preparation of **4**. The requisite tri-aldehyde for the synthesis of the poly-nitrone **1**, *i.e.* the 1,3,5-triformylbenzene, was prepared following a two-step synthesis that has been previously described.[8] Poly-nitrones **1**, **3** and **4** were purified by recrystallisation, while **2** was purified by flash-chromatography on a silica gel column, using diethyl ether–methanol (98 : 2; v/v) as the elution mixture. The synthesis yields indicated hereafter are those calculated *after* purification. All the compounds obtained were identified by microanalysis and on the basis of their ^1H, ^{13}C and ^{31}P NMR spectra, recorded on Bruker AC 100 (^1H, 100 MHz; ^{31}P, 40.53 MHz), Bruker AC 200 (^1H, 200 MHz; ^{13}C, 50.32 MHz), and Bruker AM 400X (^1H, 400 MHz; ^{13}C, 100.61 MHz) spectrometers. The chemical shifts (δ) in ppm are reported with respect to internal TMS for ^1H and ^{13}C NMR, and to external 85% H_3PO_4 for ^{31}P NMR. *J* values are given in Hz.

1,3,5-Tris[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxido-iminiomethyl]benzene, 1. Recrystallised in ethyl acetate. Yield, 64%; mp 163 °C. Elemental analysis calculated for $C_{22}H_{38}N_2O_8P_2$ (741.670): C, 48.58; H, 7.34; N, 5.67; found: C, 48.32; H, 7.44; N, 5.52%. δ_H ($CDCl_3$, 100 MHz) 9.44 (3H, s, *H* arom.), 7.75 (3H, d, ^4J_P 2.5, *H*C=N), 4.20 (12H, qt, ^3J_H 7.4, ^3J_P 7.4, O-CH_2), 1.82 (18H, d, ^3J_P 14.8, P-C-CH_3), 1.31 (18H, t, ^3J_H 7.0, O-CH_2-CH_3); δ_C ($CDCl_3$, 50.32 MHz) 132.63 (3 C, d, ^3J_P 5.8, H*C*=N), 131.18 (3C, s, *C*H arom.), 130.91 (3C, s, *C* arom.), 73.56 (3C, d, ^1J_P 155.8, *C*-P), 63.42 (6C, d, ^2J_P 7.1, O-*C*H_2), 23.45 (6C, s, *C*H_3), 16.51 (6C, d, ^3J_P 5.7, O-CH_2-*C*H_3); δ_P ($CDCl_3$) 23.1.

1,3-Bis[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxido-iminiomethyl]benzene, 2. Yield, 89%. Oil. Elemental analysis calculated for $C_{22}H_{38}N_2O_8P_2$ (520.488): C, 50.76; H, 7.36; N, 5.38. Found: C, 51.33; H, 7.83; N, 5.63%.

δ_H ($CDCl_3$, 400 MHz) 9.06 (1H, s, *H* arom.), 8.43 (2H, d, ^3J_P 7.8, *H* arom.), 7.70 (2H, d, ^4J_P 2.8, *H*C=N), 7.46 (1H, t, J_H 7.8, *H* arom.), 4.17 (8H, qt, ^3J_H 7.0, O-CH_2), 1.82 (12H, d, ^3J_P 14.9, P-C-CH_3), 1.30 (12H, t, ^3J_H 7, O-CH_2-CH_3); δ_C ($CDCl_3$, 100.61 MHz) 132.85 (2C, d, ^3J_P 5.1, H*C*=N), 130.60 (2C, s, *C*H arom.), 130.78 (2C, s, *C* arom.), 129.92 (1C, s, *C*H arom.), 128.77 (1C, s, *C*H arom.), 73.18 (2C, d, ^1J_P 155.1, *C*-P), 63.43 (4C, d, ^2J_P 6.8, O-*C*H_2), 23.37 (4C, s, *C*H_3), 16.45 (4C, d, ^3J_P 5.9, O-CH_2-*C*H_3); δ_P ($CDCl_3$) 25.7.

1,4-Bis[*N*-(1-diethylphosphono-1-methylethyl)-*N*-oxido-iminiomethyl]benzene, 3. Recrystallised in diethyl ether–pentane (1 : 1). Yield, 65%. Mp 134 °C. Elemental analysis calculated for $C_{22}H_{38}N_2O_8P_2$ (520.488): C, 50.76; H, 7.36; N, 5.38. Found: C, 50.44; H, 7.46; N, 5.19%. δ_H ($CDCl_3$, 200 MHz) 8.27 (4H, s, *H* arom.), 7.75 (2H, d, ^3J_P 2.8, *H*C=N), 4.18 (8H, qt, ^3J_P 7.1, ^3J_H 7.1, O-CH_2), 1.82 (12H, d, ^3J_P 14.8, C-CH_3), 1.30 (12H, t, ^3J_H 7.1, O-CH_2-CH_3); δ_C ($CDCl_3$, 100.61 MHz) 132.73 (2C, d, ^3J_P 4, H*C*=N), 132.10 (2C, s, *C* arom.), 128.91 (4C, s, *C*H arom.), 73.17 (2C, d, ^1J_P 154.1, *C*-P), 63.51 (4C, d, ^2J_P 6.9, O-*C*H_2-CH_3), 23.32 (4C, s, C-*C*H_3), 16.48 (4C, d, ^3J_P 5.7, O-CH_2-*C*H_3); δ_P ($CDCl_3$) 23.6.

1,2-Bis[*N*-(1-diethylphosphono)-1-methylethyl)-*N*-oxido-iminiomethyl]benzene, 4. Recrystallised in diethyl ether–pentane (1 : 1). Yield, 65%. Mp 58 °C. Elemental analysis calculated for $C_{22}H_{38}N_2O_8P_2 \cdot 2H_2O$ (556.28): C, 47.47; H, 7.60; N, 5.03. Found: C, 47.17; H, 7.64; N, 4.98%. δ_H ($CDCl_3$, 100 MHz) 8.52 (2H, m, *H* arom.), 7.71 (2H, d, ^4J_P 2.5, *H*C=N), 7.16 (2H, m, *H* arom.), 3.94 (4H, qt, ^3J_P 7.1, ^3J_H 7.1, O-CH_2), 1.59 (12H, d, ^3J_P 14.9, P-C-CH_3), 1.06 (12H, t, ^3J_H 7.1, O-CH_2-CH_3); δ_C ($CDCl_3$, 100.61 MHz) 130.10 (2C, d, ^3J_P 5.1, H*C*=N), 129.86 (2C, s, *C*H arom.), 128.87 (2C, s, *C* arom.), 128.34 (2C, s, *C*H arom.), 73.75 (2C, d, ^1J_P 154.3, *C*-P), 63.43 (4C, d, ^2J_P 6.8, O-*C*H_2), 23.44 (4C, s, P-C-*C*H_3), 16.44 (4C, d, ^3J_P 5.9, O-CH_2-*C*H_3); δ_P ($CDCl_3$) 24.76.

K_p Determination

The lipophilicity of **1**–**4** was evaluated from their partition coefficients K_p in *n*-octanol–phosphate buffer (0.1 mol dm^{-3}, pH 7) following a method described previously.[6c,10] Solutions of poly-nitrones were prepared in *n*-octanol at a concentration of 0.25 mol dm^{-3}. Equal volumes of freshly prepared octanolic solution of poly-nitrone and of phosphate buffer were mixed and vigorously stirred at 37 °C for 1 h, and the two phases were separated by brief centrifugation (1000 *g* for 20 s). The poly-nitrone concentration in either the octanolic or the aqueous phase was determined by HPLC, by using a Waters 996 PAD, a Waters 717 plus autosampler, and a Waters 600 pump. Data handling was accomplished using the Millenium 32 software. HPLC column conditions were as follows: Kromasil 5 μm C18 column (25 cm length, 4.6 mm id); flow rate, 1 cm^3 min^{-1}; injection volume, 10 mm^3; isocratic elution solvent, 70% methanol, 30% water. A 3×10^{-2} mmol dm^{-3} acetophenone solution was used as internal standard. For each poly-nitrone tested, K_p was evaluated as the ratio of the poly-nitrone concentration in *n*-octanol to that in phosphate buffer.

Spin-trapping studies

EPR assays were carried out at room temperature in capillary tubes by using a computer-controlled Bruker EMX spectrometer operating at X-band with 100 kHz modulation frequency. For the various spin adducts, the hfcc values were determined by EPR signal simulations using the computer program elaborated by Duling.[15] All spin-trapping experiments were performed in 0.1 mol dm^{-3} phosphate buffer (pH 5.8 and 7.2) in the presence of 5 mmol dm^{-3} poly-nitrone, unless otherwise stated.

Hydroxyl radical was produced in the presence of a poly-nitrone using a standard Fenton system (5 mmol dm^{-3} $FeSO_4$, 5 mmol dm^{-3} EDTA, and 2 mmol dm^{-3} H_2O_2). The EPR spectra were recorded 40 s after the addition of hydrogen peroxide. No signal was observed when catalase (400 unit cm^3)

was added before H_2O_2. Alternatively, the aminoxyl radicals TN–OH, MDN–OH and PDN–OH were generated by nucleophilic addition of water to **1**–**3** (10 mmol dm^{-3}) in the presence of $FeCl_3$ (1 mmol dm^{-3}).

The carbon-centred radicals $^{\bullet}CH_2OH$, $^{\bullet}CH_3$, and $^{\bullet}CO_2^-$ were generated in the presence of the spin traps studied by addition of methanol (15%), dimethyl sulfoxide (15%), or sodium formate (0.2 mol dm^{-3}), respectively, to the standard Fenton system described above.

Two superoxide generators were employed. The first was the light–riboflavin–electron donor (LRED) system, obtained by irradiating a solution containing 0.8 mol dm^{-3} riboflavin and 3 mol dm^{-3} ethylenetriaminepentaacetic acid (DTPA) with blue light. The second system contained 1.6 mmol dm^{-3} xanthine and 0.4 unit cm^{-3} xanthine oxidase (X–XO system). In both cases, oxygen was bubbled into the medium before irradiation or before the addition of xanthine oxidase. Whatever the superoxide generator, no signal was observed when the experiments were carried out in the presence of superoxide dismutase (SOD, 600 unit cm^{-3}).

Decay kinetics of superoxide adducts

The X–XO system described previously was used to generate superoxide in the presence of a poly-nitrone. SOD (1200 unit cm^{-3}) was added to the reaction 10 min after the the reaction had begun. The medium was then transferred into EPR tubes and the signal was recorded over 335–671 s in order to observe the superoxide adduct decay in a single spectrum. These spectra were simulated by the computer program elaborated by Rockenbauer and Korecz,[14] which permitted calculation of the rate constants for the first-order decay of the superoxide adducts.

Kinetic study of $^{\bullet}CH_3$ trapping

The Fenton reaction system in the presence of DMSO was employed to generate methyl radicals. The method of kinetic competition permitted evaluation of the ratio of the second-order rate constants for the trapping of $^{\bullet}CH_3$ by a poly-nitrone (k_N) and POBN (k_{POBN}), used as a competitive inhibitor. The concentrations of the poly-nitrone considered and of POBN were varied from 1 to 10 mmol dm^{-3}, in such a way that the ratio of the poly-nitrone N concentration to that of POBN, *i.e.* [N]/[POBN], was kept between 0.1 and 3.33. EPR signals were recorded exactly 1 min after the reaction had begun.

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